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Knockdown of BCL2L12 leads to cisplatin resistance in MDA-MB-231 breast cancer cells

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ABSTRACT

BCL2L12, a newly identified member of Bcl-2 family, contains a BH2 domain and a putative BH3 domain. It was found to be highly expressed in normal breast tissues, and was associated with favorable prognosis in breast cancer patients. Here, we reported that the mRNA levels of BCL2L12 and its transcript variant BCL2L12A could be upregulated upon cisplatin treatment in MDA-MB-231 breast cancer cells. Knockdown of BCL2L12 and BCL2L12A dramatically inhibited cisplatin-induced apoptosis. In contrast, ectopic expressions of each of the proteins promoted cisplatin-induced apoptosis. These results indicated that decreased expressions or loss of BCL2L12 and BCL2L12A may contribute to the cisplatin resistance in breast cancer patients. Furthermore, we found that cisplatin-induced downregulation of β-catenin was partially suppressed in BCL2L12- and BCL2L12A-knocked down MDA-MB-231 cells, which indicated that knockdown of these two proteins may stabilize β-catenin in cisplatin-induced apoptosis. In short, we proposed that BCL2L12 and BCL2L12A may play an important role in cisplatin-induced apoptosis in MDA-MB-231 breast cancer cells.

1. Introduction

Breast cancer is the most ubiquitous malignancy among women, and therefore an important public health issue [1]. It is expected to account for 26% (182,460) and rank the first of all new cancer cases among American women, and accounts for 15% of all female cancer deaths [2]. Among the wide variety of drugs used in breast cancer chemotherapy regimens is doxorubicin that possesses a broad spectrum of anti-tumor activity, cisplatin, and carboplatin [3–5].

Cisplatin (cis-diamminedichloroplatinumII, CDDP) is one of the most potent anti-tumor agents known, displaying clinical activity against a wide variety of solid tumors, including breast cancer. Its cytotoxic mode of action is mediated by the interaction with DNA to form DNA adducts, primarily intra-strand crosslink adducts, which activate several signal transduction pathways, including those involving ATR, p53, p73 and MAPK, and culminate in the activation of apoptosis [6–10].

Although cisplatin is effective against many human tumors, some are intrinsically resistant. And even among initially sensitive tumors, acquired resistance develops commonly during treatment. The rapid development of techniques for molecularly engineering cells to disrupt the expression of single genes has provided an alternative strategy that yielded novel insights into previously unsuspected mechanisms that control cisplatin sensitivity. C-Jun transcription factor AP1 in mouse and Lyn tyrosine kinase in chicken are among those genes whose knockout may lead to cisplatin resistance [11]. Several other genes were also found to be associated with cisplatin sensitivity. AKT was reported to promote cisplatin resistance in human ovarian cancer cells, while c-myc sensitizes colon cancer cells SW480 and SW620 to cisplatin-induced apoptosis [12,13]. And as a well established anti-tumor protein, p53 plays a central role in chemotherapy-induced apoptosis; however, its importance as a determinant of cisplatin sensitivity differs in various cell lines and tissues [11]. In all, cisplatin resistance is cell type and tissue dependent and the clinical mechanisms that lead to cisplatin resistance are complex, which means a large amount of work remains to be done.

Upstream factors involved in the cellular response to DNA damage mediate the induction of a network that transmits both pro- and anti-apoptotic signals. Any interference that favors anti-apoptotic signal transduction or abrogates pro-apoptotic pathways, including the transcriptional and translational responses, is a potential mechanism of cisplatin resistance [14]. Apoptotic events are regulated by a number of proteins that exert either positive (pro-apoptotic) or negative (anti-apoptotic) effects on programmed cell death. Proteins participating in these events include members of the Bcl-2 family which are characterized by the presence of at least one of the BH1, BH2, BH3 and BH4 domains. The BH1 and BH2 domains are present in all anti-apoptotic proteins, while the BH3 domain is present in the

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pro-apoptotic proteins as well as some anti-apoptotic proteins, such as Bcl-2 and Bcl-XL [15–17]. Most apoptosis-related genes regulate cellular fate as a response to anticancer drugs, and the levels of various Bcl-2 family proteins have been shown to determine the responses of tumor cells to chemotherapies [18,19].

BCL2L12 is a newly identified member of Bcl2 family. BCL2L12 has two splicing variants: the classical form of the gene and a truncated form called BCL2L12A which lacks exon 3 (143 bp) [20]. BCL2L12 was predicted to contain a highly conserved BH2 domain and a BH3-like motif which contributed to its pro-apoptotic activity in UV treated cells [20,21]. BCL2L12A, although lacked the BH2 domain, shared exactly the same sequence with BCL2L12 from the fist amino acid to the 120th amino acid. BCL2L12 and BCL2L12A were highly expressed in normal breast tissues [20]. Statistic analysis revealed that BCL2L12-positive breast tumors were mainly of lower stage (I/II) or grade (I/II) and that breast cancer patients with positive BCL2L12 expression were almost 5 times less likely to relapse or die, which confirmed an association of BCL2L12 with favorable prognosis [22]. But the underlying mechanisms remain to be studied.

In the present study, we intended to clarify the favorable role of BCL2L12 in breast cancer patients, and uncover the underlying molecular mechanism to this clinical phenomenon. To this end, we explored an shRNA strategy to knockdown endogenous BCL2L12. And we found that knockdown of BCL2L12 and BCL2L12A expressions with a gene specific shRNA dramatically suppressed cisplatin-induced apoptosis in MDA-MB-231 cells, while ectopic expressions of BCL2L12 or BCL2L12A promoted cisplatin-induced apoptosis. In a conclusion, our results identified that BCL2L12 and BCL2L12A promoted cisplatin-induced apoptosis in MDA-MB-231 breast cancer cells and suggested that knockdown of these two proteins may stabilize β-catenin in cisplatin-induced apoptosis.

2. Materials and methods

2.1. Chemicals and reagents

Fetal calf serum, Trizol Reagent and Lipofectamine reagent were purchased from Invitrogen. DMEM medium and cisplatin were from Sigma Chemical. Cisplatin was dissolved in PBS at a concentration of 10 mM and diluted to indicated concentrations when inducing apoptosis. Anti-Bad, anti-Bid, anti-Bax, anti-Bak, anti-Bcl-2 and anti-PARP antibodies were purchased from Cell Signaling Technology. Anti-EGFP antibody was purchased from Roche Applied Science. Anti-GAPDH antibody was purchased from KangChen Bio-tech. Chemiluminescence (ECL) assay kit was purchased from Tiangen Biotech Company.

2.2. Plasmid construction

The first exon of human BCL2L12 was isolated from genome DNA of human lung cell line PG-BE1 by PCR with primers (5′-ATACTCGAGATGGGACGGCCCGCTGGGCTG-3′ and 5′-GCCATGGAGG-CACCTGGTCCGGGGCCCCAC-3′). The rest of BCL2L12's open reading frame was amplified from brain cDNA library with primers (5′-GGGGCCCCGGACCAGGTGCCTCCATGGC-3′ and 5′-ATAGAATTCCG-TCCAATGGCAAGTTCAAG-3′). Then the entire cDNA of BCL2L12 was amplified using primers (5′-AAAGAATTCATGGGACGGCCCGCT GG-3′ and 5′-GTGCTCGAGTCAGTCCAATGGCAAGTTC-3′) and cloned into pcDNA3.0-GFP [23]. For knockdown of BCL2L12 and BCL2L12A expressions, two complementary oligonucleotides targeted to the BCL2L12 gene were designed: 5′-GATCCGGAAGCCATACTGCGGAGGTTCAAGAGACCTCCG-CAGTATGGCTTCCTTTTTTGGAAA-3′ and 5′-AGCTTTTCCAAAAAAGGAA-GCCATACTGCGGAGGTCTCTTGAACCTCCGCAGTATGGCTTCCG-3′. Plasmid pSi-BCL2L12 was constructed by inserting the annealed complementary oligonucleotides into the pSilencer2.1-U6-neo vector (Ambion). All constructs were confirmed by DNA sequencing.

2.3. Cell culture, transfection and western blot

MDA-MB-231 breast cancer cells were cultured in Dulbecco's modified Eagle's medium (DMEM) and supplemented with 10% fetal bovine serum. MCF-7 breast cancer cells were cultured in 10% FBS–DMEM and supplemented with 0.01 mg/ml bovine insulin. Cell transfection was performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. For stable transfection, 48 h after transfection, the cells were selected in the DMEM containing G418 (400 μg/ml). After 2–3 weeks' growth in G418-containing medium the individual G418-resistant clones were selected, expanded and validated.

2.4. Western blot analysis

Western blot experiments were used to measure certain proteins. Briefly, the cells were lysed in 1× SDS lysis buffer (40 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% SDS, 1 mM aprotinin, 1 mM PMSF and 10 μg/ml Leupeptin). A total of 50 μg of protein from each sample was electrophoresed by 8% or 10% SDS-PAGE and then transferred to a PVDF membrane. After blocking with PBS containing 5% nonfat milk and 0.1% Tween 20 overnight, the membrane was incubated with primary antibody at room temperature for 2 h. After washing with PBS containing 0.1% Tween 20 three times, each for 10 min, the membrane was then incubated with horseradish peroxidase labeled secondary antibody for another 1 h at room temperature. The membrane was then developed using the enhanced chemiluminescent (ECL) detection system.

2.5. Analysis of apoptosis

2.5.1. Analysis of nuclear morphology by fluorescence staining

Cells grown on the glass coverslips were fixed with 4% paraformaldehyde/PBS for 30 min, washed for 15 min in 0.1% Triton X-100/PBS, and incubated in dark with Hoechst 33258 (20 μg/ml) for 15 min. After the coverslips were washed in PBS, positive nuclei were counted. Normal nuclei and apoptotic nuclei (condensed new moon-type or fragmented chromatin) were easily distinguished. A minimum of 300 cells from three different microscopic fields were counted to obtain reliable estimates of cell apoptosis in three independent experiments.

2.5.2. Apoptosis of GFP-positive cells was assessed by PI staining

24 h after transfection, the cells were treated with indicated amounts of cisplatin for 2 h and incubated in fresh media for another 24 h. Then, the adherent and non-adherent cells were collected, washed twice in phosphate-buffered saline (PBS), fixed with 4% PFA, and permeated in 0.1% Triton X-100/PBS on ice for 10 min. The fixed cells were washed and stained with propidium iodide mixture containing 50 μg/ml propidium iodide, 0.05% Triton X-100, 37 μg/ml EDTA, and 100 U/ml ribonuclease in PBS. After incubation for 45 min at 37 °C, the DNA content was determined by quantitative flow cytometry with standard optics of FACScan flow cytometer (Becton-Dickinson FACStar), and GFP-positive cells were gated and the percentage of apoptosis was quantitated from sub-G1 events.

2.5.3. Apoptosis analysis by annexin-V and PI staining

This analysis was performed as described before [24]. Briefly, adherent and non-adherent cells were collected, washed twice in phosphate-buffered saline (PBS), and then resuspended in binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, and 2.5 mM CaCl2). 195 μl of cell suspension containing 10^5 cells was taken, and 5 μl of annexin-V–FITC was added, mixed, and incubated for 10 min in the dark. After that, the cells were washed twice in PBS and resuspended in 190 μl of binding buffer. 10 μl of 20 μg/ml PI was added and then analyzed by fluorescence-activated cell sorting. Cells in the early stage of apoptosis could be stained with annexin-V but not PI. The necrotic and late stage of apoptotic cells were stained by both annexin-V–FITC and PI.

2.6. Total RNA extraction and reverse transcription (RT)-PCR

Total mRNA samples of MDA-MB-231 breast cancer cells were extracted using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Samples (1.0 μg) were used as templates to perform the RT-PCR assay. The RT-PCR and real-time PCR was performed by using TaKaRa RNA PCR Kit (AMV) Ver. 3.0 and SYBR Premix Ex Taq II according to the manual (TaKaRa), respectively. The cDNA was subjected to denaturation at 95 °C for 5 min, followed by 30 cycles (94 °C for 30 s, 57 °C for 30 s, and 72 °C for 45 s) and incubated at 72 °C for 10 min and 4 °C for 5 min. Then products were applied to 1.5% agarose gel electrophoresis. Primers used for conventional PCR were as follows: BCL2L12-F 5′-TTCCGCCCTTTCTACGCTGG-3′ and BCL2L12-R 5′-GCCTCCGCAGTATGGCTTCC-3′; β-actin-F 5′-ATGGATGATGATATCGCCGC-3′ and β-actin-R 5′-CATCACGATGCCAGTGGTAC-3′. And the densitometric programme used was Totallab 2.01. Primers used for real-time PCR were as follows: L12-real-time-F 5′-AAGCCCTGCCCAAGAAGAGCCA-3′, L12-real-time-R 5′-CCAGGCTCTAAACCATAGCAGGG-3′; Real-time RT-PCR was performed using an iCycler iQ multicolor real-time PCR detection system (Bio-Rad) with the following cycling

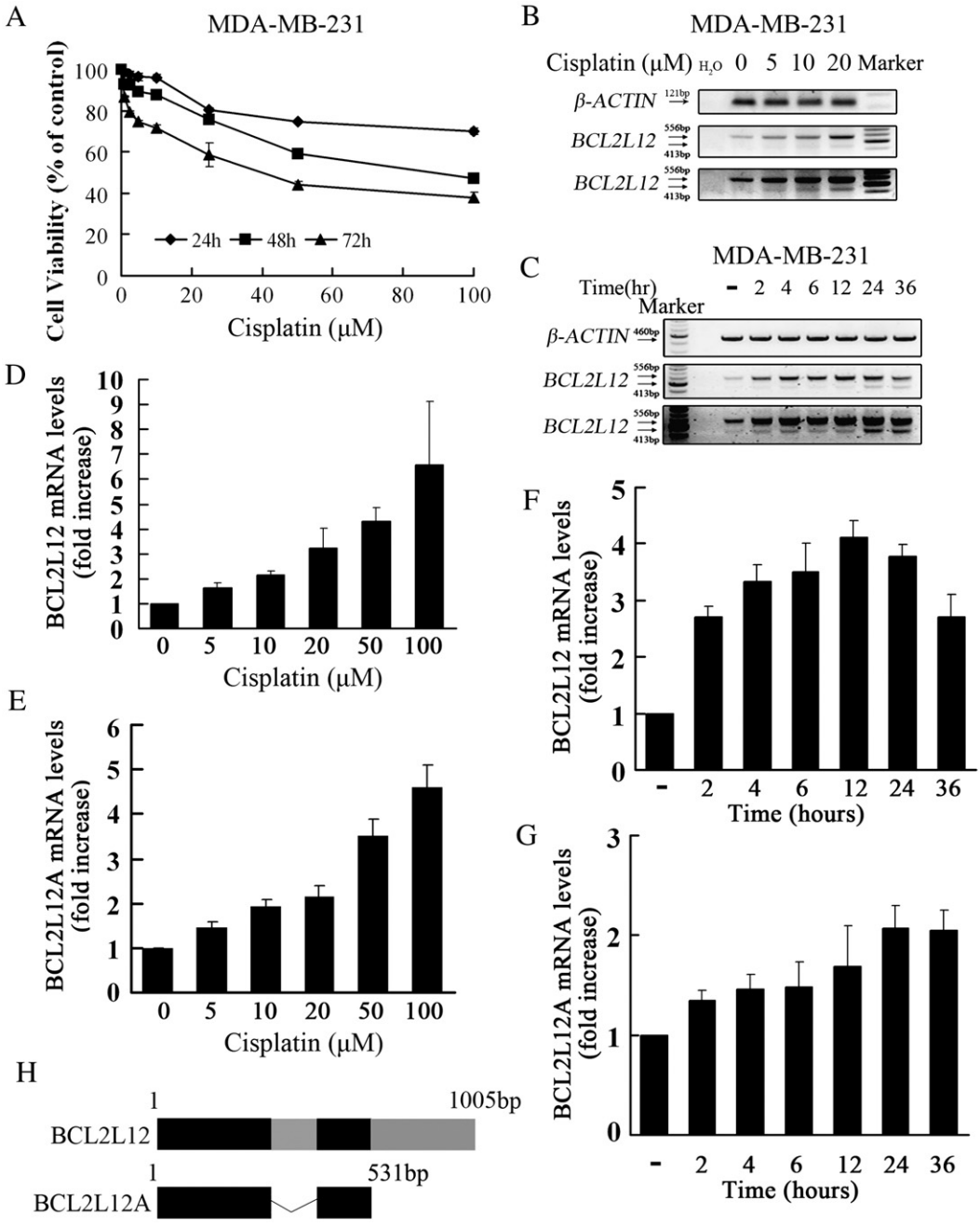

Fig. 1. BCL2L12 and BCL2L12A mRNA were upregulated upon cisplatin treatment in MDA-MB-231 breast cancer cell. (A) MDA-MB-231 cells were exposed to increasing doses of the drug (0, 1, 2.5, 5, 10, 25, 50, and 100 μM) for increasing periods of time (24, 48, and 72 h), and cell viability was measured by the MTT assay. (B) MDA-MB-231 cells were treated with indicated amount of cisplatin for 2 h and then incubated in fresh media for 24 h. (C) MDA-MB-231 cells were treated with 10 μg/ml cisplatin for indicated times. (D and F) The BCL2L12 mRNA level was further quantified by real-time PCR analysis. (E and G) The BCL2L12A mRNA level was quantified by a densitometric programme named Totallab based on the electrophoresis results. Total cellular RNA was isolated using trizol reagent, and 1 μg RNA was used to perform RT-PCR analysis to assess the mRNA levels of BCL2L12 and BCL2L12A. β-actin was used as an internal control. The data were presented as increases (*n*-fold) compared to the PBS-treated samples. Each bar represents the mean ± SD of three independent experiments. While BCL2L12A levels were relatively low in the middle panel of Fig. 1B and C, we increased the PCR cycles from 30 to 35, and used a higher intensity of UV light to get a distinguishable vision of BCL2L12A in the lower panels.

conditions:(i) 15 s at 94 °C and (ii) 50 cycles, with 1 cycle consisting of 15 s at 94 °C, 20 s at 56 °C, and 15 s at 72 °C. β-actin was employed as an internal reference under the same experimental conditions. Data were analyzed by using iCycler iQ software (Bio-Rad). The values were obtained through normalizing BCL2L12 copies to β-actin copies.

2.7. Cell viability assay

Relative cell viability was determined by MTT assay. Cells (1×10^4 cells/well) were plated in 96-well microtiter plates. Twenty four hours after transfection, cells were incubated with different concentrations of cisplatin for another 24 h at 37 °C. MTT was added to the culture medium to yield a final MTT concentration of 0.5 mg/ml following cell treatment and the incubation was continued for 4 h at 37 °C. The pellets were dissolved with dimethyl sulfoxide at room temperature for 10 min. Cell viability was determined by measuring the absorbance of the converted dye at a wavelength of 490 nm.

3. Results

3.1. BCL2L12 mRNA and BCL2L12A mRNA were upregulated upon cisplatin treatment in MDA-MB-231 breast cancer cells

As BCL2L12 and BCL2L12A were highly expressed in normal breast tissues and associated with a favorable prognosis in breast cancer patients [20,22], we investigated their functions in breast cancer cells and their contributions to cisplatin-based chemotherapy. To determine the sensitivity of MDA-MB-231 cells to cisplatin, the cells were exposed to increasing doses of the drug (0, 1, 2.5, 5, 10, 25, 50, 100 μM) for increasing periods of time (24, 48, and 72 h), and cell viability was measured by the MTT assay (Fig. 1A). We then examined the expressions of BCL2L12 and BCL2L12A in MDA-MB-231 breast cancer cells treated with cisplatin (CDDP). RT-PCR was performed to analyze the mRNA levels of both proteins in vehicle (PBS)- or CDDP-treated MDA-MB-231 cells. As depicted in Fig. 1B, the mRNA levels of both proteins were significantly increased upon CDDP treatment in a dose-dependent manner. In order to detect the mRNA levels of BCL2L12 and BCL2L12A treated with cisplatin in different times, the cells were first treated with 20 μM cisplatin for 2 h and then incubated in fresh media for the indicated times. As the results shown in Fig. 1C, both the mRNA levels of BCL2L12 and BCL2L12A were upregulated in a time-dependent manner before 24 h and downregulated afterwards (Fig. 1C). The BCL2L12 mRNA level was further quantified by real-time PCR analysis (Fig. 1D and F), which gave a similar result as the conventional PCR method. The BCL2L12A mRNA level was quantified by a densitometric programme named Totallab based on the electrophoresis results (Fig. 1E and G). BCL2L12A is a splicing variant of BCL2L12, and BCL2L12 shares all the sequences BCL2L12A has (Fig. 1H). The primers designed for the detection of BCL2L12 in real-time PCR can only recognize BCL2L12 but not BCL2L12A. However, it may not be possible for us to design such pair of primers for BCL2L12A in real-time PCR. So we can only quantify the levels of BCL2L12A with the conventional PCR.

3.2. shRNA-mediated knockdowns of BCL2L12 and BCL2L12A in MDA-MB-231 breast cancer cells

The fact that BCL2L12 and BCL2L12A mRNA were upregulated dramatically upon cisplatin treatment in MDA-MB-231 cells drove us to investigate whether the elevated expressions of both proteins were associated with CDDP-induced apoptosis. To do so, we employed an shRNA approach to knockdown the endogenous BCL2L12 and BCL2L12A. Two complementary oligonucleotides targeted to the BCL2L12 gene were designed, denatured and inserted into pSilencer2.1-U6-neo plasmid. Then, pSilencer2.1-U6-BCL2L12 (pSi-BCL2L12) and mock vector were transfected into MDA-MB-231 cells respectively, and the cells stably expressing pSi-BCL2L12 and mock vector were selected using G418 (400 μg/ml). The total cellular RNAs of stably transfected clones were subjected to analyze the mRNA levels of both proteins. As shown in Fig. 2A, B and C, the expressions of these two proteins were downregulated in pSi-BCL2L12-expressing cells. In order to test the specificity of pSi-BCL2L12, we also analyzed the expressions of other Bcl-2 family members, such as Bcl-2, Bcl-xl, Bax, Bim and Bad (Fig. 2E and F), which remained unchanged in MDA-MB-231/pSi-BCL2L12 cells. To visualize the reduction of BCL2L12 expression in the protein level, we further transfected cells with a GFP-BCL2L12 fusion gene. The expression of GFP-BCL2L12 was reduced by approximately 80% in cells transfected with pSi-BCL2L12 compared to that in control cells (Fig. 2D). The expression of GFP was used as a negative control. These results demonstrated that the expressions of BCL2L12 and BCL2L12A in MDA-MB-231 breast cancer cells were suppressed by pSi-BCL2L12 with high efficiency and specificity.

3.3. Knockdown of BCL2L12 and BCL2L12A resulted in elevated resistance of MDA-MB-231 to CDDP-induced apoptosis

Having established that BCL2L12 and BCL2L12A expressions were reduced significantly by pSi-BCL2L12, we analyzed whether the downregulation of these two proteins would affect the sensitivity of MDA-MB-231 cells to CDDP-induced apoptosis. To do so, MDA-MB-

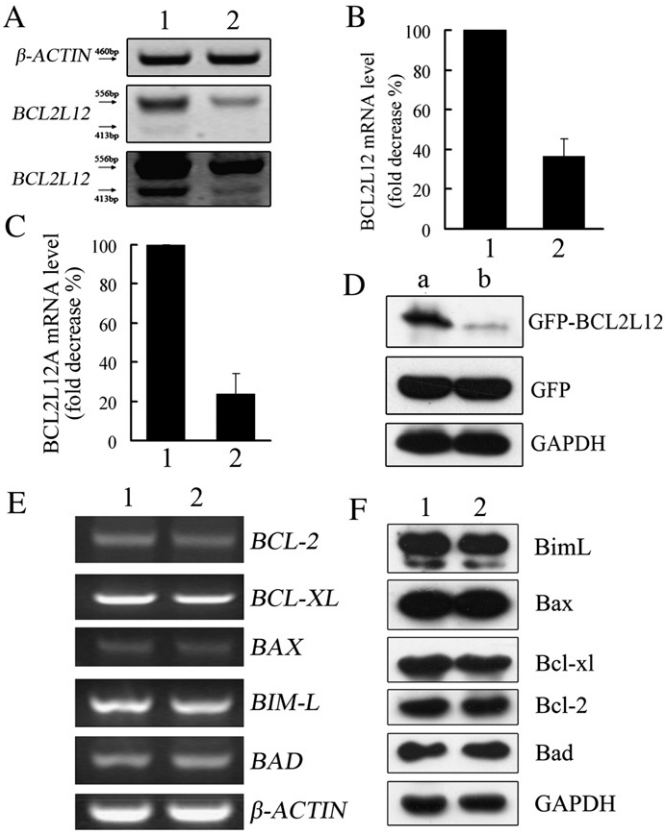

Fig. 2. shRNA-mediated knockdown of BCL2L12 and BCL2L12A in MDA-MB-231 breast cancer cells. (A) shRNA-mediated knockdown of BCL2L12. MDA-MB-231 cells were transfected with pSilencer 2.1 or pSi-BCL2L12 respectively. The stable cell lines were chosen as mentioned earlier. The total RNA of each clone was extracted and subjected to RT-PCR analysis of BCL2L12 and BCL2L12A mRNA levels. (B) Quantification of BCL2L12 mRNA level with real-time PCR. (C) Quantification of BCL2L12A mRNA level using Totallab 2.01. (D) Inhibition of BCL2L12 protein expression. GFP-BCL2L12 was co-transfected with GFP and pSilencer2.1 (a) or pSi-BCL2L12 (b) into MDA-MB-231 cells. Cell lysates were subjected to immunoblot analysis with antibody to GFP. GFP was used as a negative control and GAPDH as a loading control. (E, F) The mRNA levels and protein levels of other Bcl-2 family members in cells stably transfected with pSilencer2.1 or pSi-BCL2L12 were analyzed.

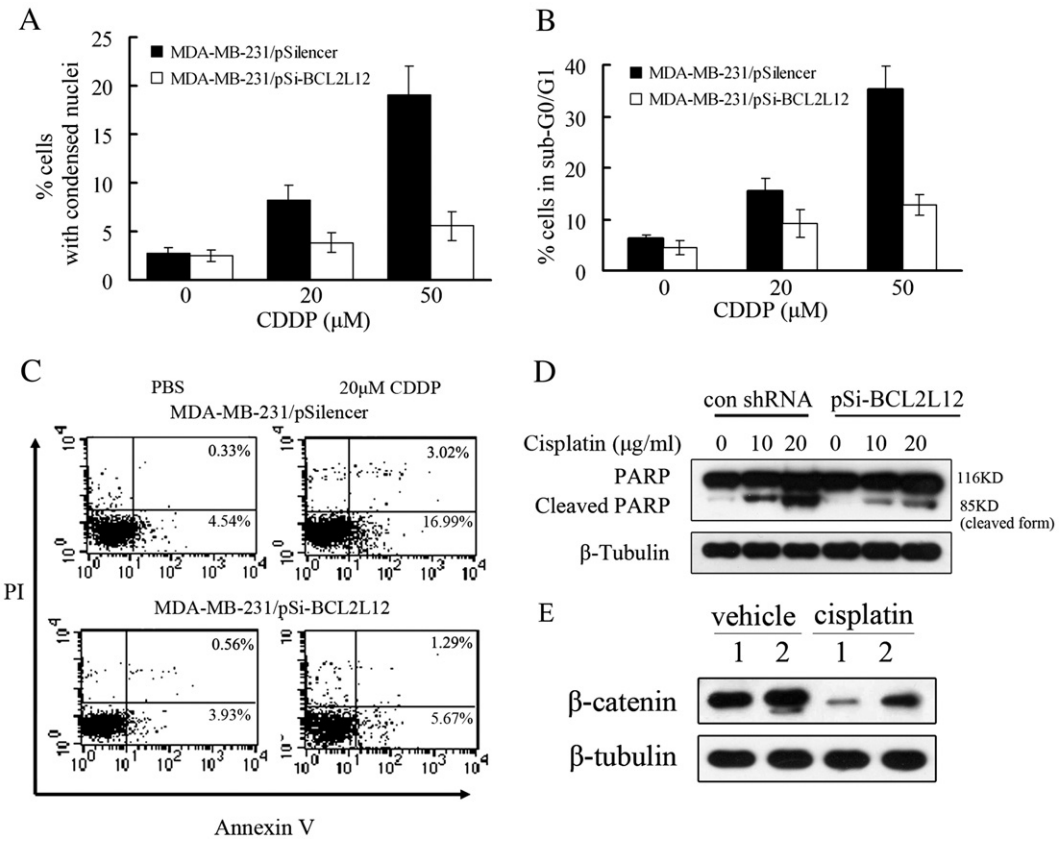

Fig. 3. Knockdown of BCL2L12 and BCL2L12A resulted in elevated resistance of MDA-MB-231 to CDDP-induced apoptosis. (A) Cells stably transfected with mock vector or pSi-BCL2L12 were treated with 20 μM or 50 μM CDDP for 2 h, and then incubated in fresh media for another 24 h. After treatment, the adherent cells were stained with Hoechst 33258. At least 300 cells were counted from three different microscopic fields and each value was mean ± SD of three independent experiments. (B) Cells were harvested, stained with propidium iodide, and then subjected to flow cytometry analysis ($n=3$; $p<0.05$). (C) Cells were treated with PBS or 20 μM cisplatin for 2 h, and then incubated in fresh media for another 24 h. Adherent and non-adherent cells were harvest, washed with PBS and stained with Annexin-V and PI as described above. (D) Western blot analysis of PARP cleavage in total cell lysates of MDA-MB-231 cells stably transfected with mock vector or pSi-BCL2L12 and treated with vehicle (PBS) or cisplatin of indicated concentrations for 24 h. (E) Effect of pSi-BCL2L12 on β-catenin protein level. MDA-MB-231/pSilencer (1) and MDA-MB-231/pSi-BCL2L12 (2) cells were treated with vehicle (PBS) or cisplatin as in C. And then, cells were subjected to western blot analysis with antibodies to β-catenin and β-tubulin.

231/pSilencer or MDA-MB-231/pSi-BCL2L12 was treated with 20 μM (IC15) or 50 μM (IC30) CDDP respectively for 2 h and then incubated in fresh media for another 24 h. As measured by Hoechst staining assay (Fig. 3A), the apoptosis rates were decreased by about 40% and 60% respectively. The propidium iodide-based FACS assay also gave the similar result (Fig. 3B). The percentage of apoptotic cells was a little higher measured by PI staining compared with Hoechst staining. The reason for this may be that the cells subjected to PI-based FACS assay included both adherent and non-adherent cells, while only adherent cells were analyzed in the Hoechst staining assay. To get a better analysis of apoptosis, Annexin-V/PI double staining method was used. As shown in Fig. 3C, the percentage of cells in the early stage of apoptosis (Annexin-V+/PI−) and the percentage of cells in the late stage of apoptosis plus necrotic cells (Annexin-V+/PI+) were evidently higher in MDA-MB-231/pSilencer cells than in MDA-MB-231/pSi-BCL2L12 cells. Because PARP cleavage is a hallmark of apoptosis [25], in order to confirm the apoptosis suppression effect of pSi-BCL2L12 on MDA-MB-231 cells, we further analyzed the effect of pSi-BCL2L12 on PARP cleavage in CDDP-treated cells. As shown in Fig. 3D, the cleavage of PARP was obviously suppressed in cells stably transfected with pSi-BCL2L12. These results indicated that knockdown of BCL2L12 and BCL2L12A with pSi-BCL2L12 elevated the resistance of MDA-MB-231 cells to CDDP-induced apoptosis. In order to further investigate the signaling pathway BCL2L12 and BCL2L12A participated in cisplatin-induced apoptosis, we analyzed several apoptosis and tumor-associated proteins, such as Bcl-2 and β-catenin. Of those being examined, β-catenin was found to be differently expressed in MDA-MB-231/pSilencer and MDA-MB-231/pSi-BCL2L12 cells when treated with cisplatin. As shown in Fig. 3E, β-catenin was downregulated upon cisplatin treatment in MDA-MB-231/pSilence cells, which was suppressed in MDA-MB-231/pSi-BCL2L12 cells. This result indicated that knockdown of BCL2L12 and BCL2L12A suppressed cisplatin-induced downregulation of β-catenin. These suggested that knockdown of these two proteins might stabilize β-catenin in cisplatin-induced apoptosis. But the underlying mechanisms still remained to be studied.

3.4. Knockdown of BCL2L12 and BCL2L12A did not affect the cell cycle and cell proliferation rate of MDA-MB-231 cells

Clinical observation showed that BCL2L12 and BCL2L12A expressions were associated with and could be used as a molecular marker of favorable prognosis for breast cancer [22,26]. We considered the possibility that knockdown of BCL2L12 and BCL2L12A might affect the cellular proliferation rate of breast cancer cells. We therefore compared the growth kinetics of MDA-MB-231/pSilencer cells and MDA-MB-231/pSi-BCL2L12 cells. However, Fig. 4A showed that both cell lines gave the similar growth rates, which indicated that BCL2L12 and BCL2L12A expressions did not affect the growth of MDA-MB-231 cells. To gain further insight into whether the expressions of BCL2L12 and BCL2L12A had a subtle effect on cell growth, we made use of flow cytometry to compare the cell cycles of these two cell lines. The data

(Fig. 4B and C) showed that the distributions of MDA-MB-231/pSi-BCL2L12 cells in G1, S, and G2/M phase were not statistically distinct from that of MDA-MB-231/pSilencer cells. Taken together, we concluded that the favorable functions of BCL2L12 and BCL2L12A in MDA-MB-231 breast cancer cells might not be due to growth suppression, but by influencing the tumor's sensitivity to anti-tumor drugs.

3.5. Ectopic expressions of BCL2L12 and BCL2L12A promoted CDDP-induced apoptosis in MDA-MB-231 cells

BCL2L12 and BCL2L12A share the same sequence from the first amino acid to the 120th amino acid, but have totally different amino acid sequences from the 121st amino acid to the last ones because of the reading frame shift (Fig. 5A). BCL2L12-shRNA recognized and targeted to the C-terminal of BCL2L12 mRNA and 3′-untranslational region of BCL2L12A mRNA respectively. As shown in Fig. 2, both proteins were knocked down by pSi-BCL2L12. In order to clarify the individual effects of BCL2L12 and BCL2L12A in cisplatin-induced apoptosis, the cDNA of both genes were cloned and inserted into pcDNA3.0-GFP. Then, EGFP tagged BCL2L12 and BCL2L12A were transfected into MDA-MB-231 cells respectively (Fig. 5B, C). To testify the apoptosis-associated functions of these two proteins, 36 h after transfection, cells were treated with 20 μM cisplatin for 2 h, and then incubated in fresh media for another 24 h. Afterwards, the cells were harvested and stained with Hoechst 33258 (Fig. 5C). The statistic results showed that ectopic expressions of GFP-BCL2L12 and GFP-BCL2L12A promoted CDDP-induced apoptosis (Fig. 5D). In order to get a better result, the adherent and non-adherent cells were harvested, fixed with 4% PFA, Permeabilized with 0.1% Triton X-100, stained with PI, and then subjected to FACS analysis. GFP-positive cells were gated and the percentage of apoptosis was quantitated from sub-G1 events. Moreover, ectopic expressions of BCL2L12 and BCL2L12A markedly increased the cleavage of PARP (Fig. 5F). These results showed that both proteins could promote CDDP-induced apoptosis.

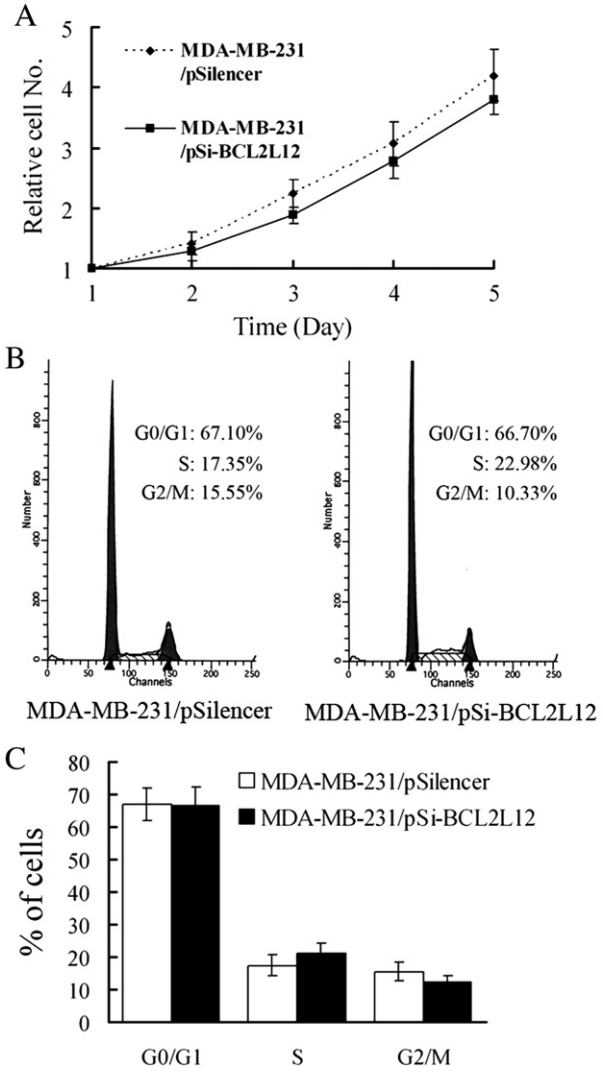

Fig. 4. Knockdown of BCL2L12 and BCL2L12A did not affect the cell cycle and cell proliferation rate of MDA-MB-231 cells. (A) Cellular proliferation analysis. 3×10^3 cells of MDA-MB-231/pSi (circle) or MDA-MB-231/pSi-BCL2L12 (triangle) were seeded and relative cell number was measured using MTT kit at the indicated times. (B) Cell cycle analysis. The distribution of each phases of asynchronous cells including MDA-MB-231/pSi and MDA-MB-231/pSi-BCL2L12 cells was quantitated by flow cytometry. The percentages of G0/G1, S, and G2/M were listed. (C) Statistical analysis of cell cycle distributions of MDA-MB-231/pSi and MDA-MB-231/pSi-BCL2L12 cells. The data shown are means±SD of three independent experiments.

4. Discussion

Bcl-2 family proteins serve as critical regulators of pathways involved in apoptosis, acting to either inhibit or promote cell death [27]. Sequence comparison of Bcl-2 family members has revealed four recurring motifs, commonly denoted Bcl-2 Homology domains (BH1 to BH4), which are indispensable in their apoptosis-associated functions [28–30]. BCL2L12 is a newly identified member of Bcl-2 family. Although it was predicted to contain a highly conserved BH2 domain and a BH3 like motif, its function in apoptosis remained elusive [20,21]. It was reported that BCL2L12 could interact with Bcl-xL and promoted UV-induced apoptosis, while Stegh, A.H. and colleagues found that BCL2L12 inhibited post-mitochondrial apoptosis signaling in glioblastoma through interacting with and neutralizing caspase-7 [21,31]. However, it is not rare for a cellular protein to exert both anti-apoptotic and pro-apoptotic functions, such as Hsp70, which protects cells from heat shock, nitric oxide exposure and many other environmental stresses, while promoting TNF-α mediated apoptosis by binding IKK-γ and impairing NF-κB survival signaling [32–34]. And even Bcl-2 itself, one of the most potent anti-apoptotic proteins, could promote apoptosis in some situations [35,36]. These findings suggested that BCL2L12 could have different functions upon exposure to different cytotoxic stimuli.

BCL2L12 can also function distinctly in different cell types. For example, overexpression of BCL2L12 in mammary tumors was correlated with improved disease-free and overall survival, while BCL2L12 expression, in particular, expression of a shorter isoform BCL2L12A, was associated with tumor progression and poor prognosis in colon cancer [22,26,37]. These findings suggested that the functions of BCL2L12 and BCL2L12A might be dependent on the cell context and may help to explain why BCL2L12 acted as an anti-apoptotic protein in glioblastoma [31], while exerted pro-apoptotic function in MDA-MB-231 breast cancer cells in our work.

Alterations of BCL2L12 mRNA level were also found in HL-60 and MCF-7 cells upon several other anti-tumor drugs treatment. Etoposide and topotecan downregulated BCL2L12 mRNA in HL-60 cells, and cisplatin and toxal downregulated BCL2L12 mRNA in MCF-7. And the mRNA level of BCL2L12 stayed stable in HL-60 and MCF-7 when methotrexate and etoposide were used respectively. Furthermore, the mRNA level of BCL2L12 was first upregulated and then downregulated in HL-60 cells when the cells were treated with cisplatin, carboplatin or doxorubicin [38–43]. Because the aim of our research was to study the BCL2L12 associated molecular mechanism of cisplatin-based chemotherapy on breast cancer, we first detect the expression of BCL2L12 in MDA-MB-231 cells in cisplatin-induced apoptosis. And we found that both the BCL2L12 and BCL2L12A mRNA levels were

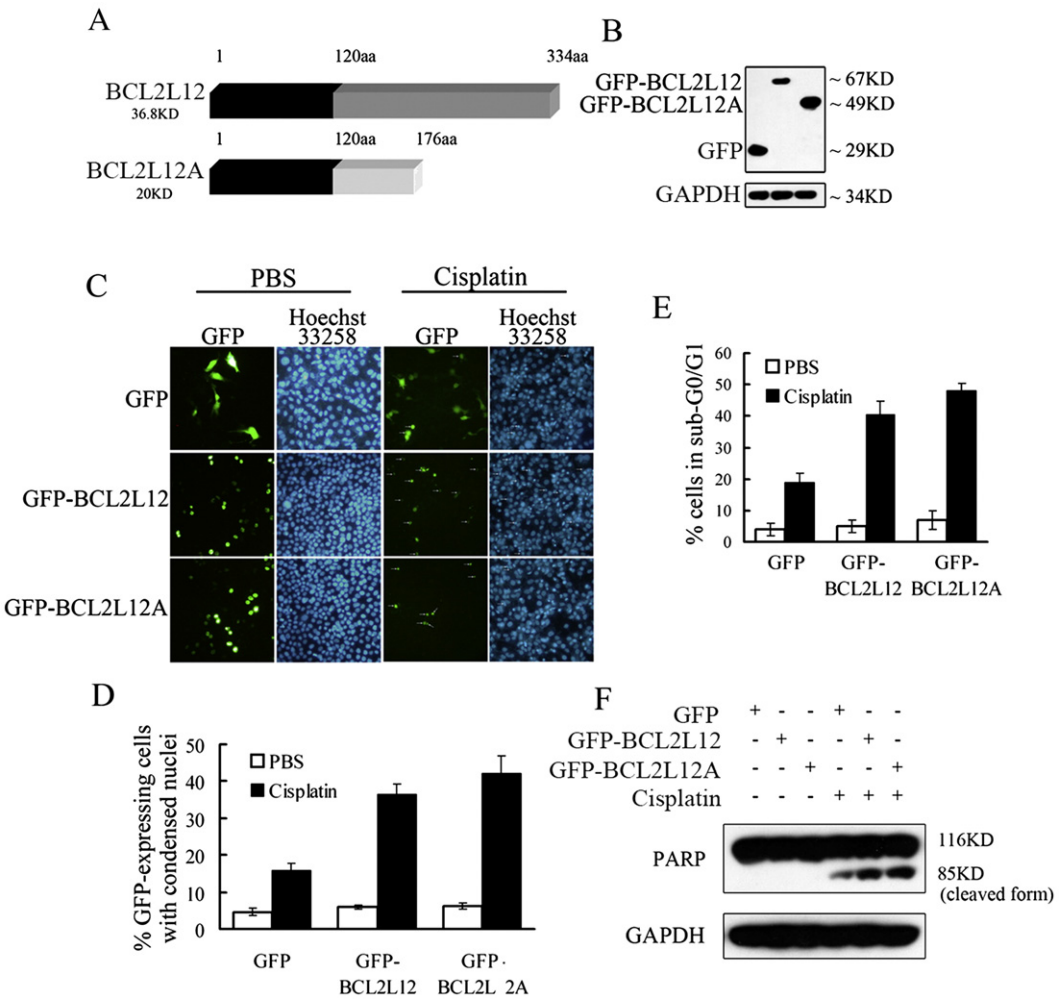

Fig. 5. Ectopic expressions of BCL2L12 and BCL2L12A promoted CDDP-induced apoptosis. (A) Schematic representations of BCL2L12 and BCL2L12A. (B) Western blot analysis of GFP, GFP-BCL2L12 and GFP-BCL2L12A expression in MDA-MB-231 cells. (C) Apoptosis was assayed morphologically. Cells transiently transfected with GFP, GFP-BCL2L12 and GFP-BCL2L12A were treated with 20 μM cisplatin for 2 h and incubated in fresh media for another 24 h. The fragrantation and condensation of apoptosis cells' nucleus were visualized by Hoechst 33258 staining. The apoptotic cells were indicated with white arrows. (D) Quantification of apoptosis rates. The apoptosis rates were counted amount GFP-positive cells, and at least 300 cells were counted from three different microscopic fields and each value was the mean ± SD of three independent experiments. (E) Cells were treated as in D, and all the adherent and non-adherent cells were harvested and subject to FACS analysis as described in the second section. (F) Western blot analysis of PARP cleavage in total cell lysates of MDA-MB-231 cells transiently transfected with GFP, GFP-BCL2L12 or GFP-BCL2L12A and treated with vehicle (PBS) or 10 μg/ml cisplatin for 24 h.

upregulated in time- and dose-dependent manners. We also made an effort to detect the mRNA levels of BCL2L12 and BCL2L12A in MCF-7 breast cancer cells upon cisplatin treatment. We found that the mRNA levels of BCL2L12 and BCL2L12A were first upregulated before 8 h, and then downregulated in the 16th hour in agreement with Thomadaki et al. where they found gene down regulation after 24 h treatment (Fig. S1-B, C and D). These alterations of BCL2L12 level during drug treatment implied a correlation between BCL2L12 and drug-induced apoptosis.

However the downregulation or upregulation of BCL2L12 itself in different cell lines and in response to different anti-tumor drugs could not directly indicate an anti-apoptotic or pro-apoptotic function of BCL2L12. For that both anti-apoptotic and pro-apoptotic proteins, such as Bcl-2 and Bax, can be downregulated in drug-induced apoptosis [41]. In order to clarify this, we explored an shRNA strategy to knock down the endogenous BCL2L12 and it splicing variant BCL2L12A. With an efficient knockdown of BCL2L12 and BCL2L12A, MDA-MB-231 cells showed an elevated resistance to cisplatin-induced apoptosis, as analyzed by Annexin-V/PI double staining and PARP cleavage. And ectopic expression of each of these proteins in MDA-NB-231 and MCF-7 cells promoted cisplatin-induced apoptosis (Fig. 5 and Fig. S1). These results implied that these two proteins might serve as pro-apoptotic proteins in cisplatin-induced apoptosis in MDA-NB-231 and MCF-7 cells.

As an anti-tumor drug, cisplatin was known to induce apoptosis of tumor cells through DNA–DNA, DNA–protein crosslink and consequent DNA damage. Adding the results proposed by Toumelin G.L. etc. that BCL2L12 promoted UV-induced apoptosis [21], we hypothesize that BCL2L12 and BCL2L12A might function as pro-apoptotic proteins in DNA damage-induced apoptosis. However, the underlying mechanisms still need to be studied, and more efforts on other DNA damage drugs and other cells should be made.

Although cisplatin-based chemotherapy is effective in treating solid tumors, the acquisition of resistance by tumor cells to cisplatin is one of the major problems in cisplatin treatment with largely unknown mechanisms [44]. In our study, we found that knockdown of endogenous BCL2L12 and BCL2L12A with a gene specific shRNA dramatically inhibit cisplatin-induced apoptosis, while ectopic expression of each of the proteins promoted cisplatin-induced apoptosis. These findings indicated that decreased expressions or loss of these two proteins may contribute to cisplatin resistance in breast cancer patients. On the contrary, upregulated expressions of both proteins might sensitize the tumor cells to cisplatin-based chemotherapy. These results may also help to explain why these two

proteins were associated with favorable prognosis in breast cancer patients.

β-catenin is a multifunctional protein involved in cell–cell adhesion and Wnt signal transduction. β-catenin signaling has been proposed to act as inducer of cell proliferation in different tumors [45]. In the nucleus, β-catenin acts as a transcriptional coactivator and activates genes involved in cell proliferation and survival [46]. Misregulation of this important protein is a causative factor in some human cancers. Downregulation of β-catenin is usually associated with apoptosis [47]. We found in our study that β-catenin was downregulated upon cisplatin treatment in MDA-MB-231 breast cancer cells, which underwent apoptosis. However, this was partially suppressed in BCL2L12- and BCL2L12A-knocked down MDA-MB-231 cells, which indicated that an elevated resistance of MDA-MB-231/pSi-BCL2L12 in cisplatin-induced apoptosis may be associated with a failure in β-catenin downregulation, and that BCL2L12 and BCL2L12A may function through destabilization of β-catenin in cisplatin-induced apoptosis.

We also found that the cell cycle distributions and growth rates were similar in MDA-MB-231/pSilencer and MDA-MB-231/pSi-BCL2L12 cells. This indicated that the favorable function of these two proteins on breast cancer supposed by Maroulio Talieri etc. may not be through cell cycle regulation but by influencing the tumor's sensitivity to anti-tumor drugs [22].

In summary, the data presented here suggested that cisplatin upregulated the expressions of BCL2L12 and BCL2L12A, which in turn promoted cisplatin-induced apoptosis in MDA-MB-231 breast cancer cells. Knockdown of BCL2L12 and BCL2L12A with a gene specific shRNA dramatically inhibited cisplatin-induced apoptosis in MDA-MB-231 cells. These findings suggested that BCL2L12 and BCL2L12A play an important role in cisplatin-induced apoptosis. Conversely, decreased expressions or loss of BCL2L12 and BCL2L12A might contribute to the cisplatin resistance in breast cancer patients.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbadis.2008.09.008.

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